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Abstract: Periodontitis is an infectious inflammatory disease that destroys the tooth-supporting tissues. It is caused by the formation of subgingival biofilms on the surface of the tooth. Characteristic bacteria associated with subgingival biofilms are the Gram-negative anaerobes Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola, collectively known as the "red complex" species. Inter-epithelial junctions ensure the barrier integrity of the gingival epithelium. This may however be disrupted by the biofilm challenge. The aim of this in vitro study was to investigate the effect of subgingival biofilms on the expression of inter-epithelial junctions by gingival epithelia, and evaluate the relative role of the red complex. Multi-layered human gingival epithelial cultures were challenged with a 10-species in vitro subgingival biofilm model, or its variant without the red complex, for 3 h and 24 h. A low-density array microfluidic card platform was then used for analysing the expression of 62 genes encoding for tight junctions, gap junctions, adherens junctions, and desmosomes. Although there was a limited effect of the biofilms on the expression of tight, adherens and gap junctions, the expression of a number of desmosomal components was affected. In particular, Desmoglein-1 displayed a limited and transient up-regulation in response to the biofilm. In contrast, Desmocollin-2, Desmoplakin and Plakoglobin were down-regulated equally by both biofilm variants, after 24 h. In conclusion, this subgingival biofilm model may down-regulate selected desmosomal junctions in the gingival epithelium, irrespective of the presence of the "red complex". In turn, this could compromise the structural integrity of the gingival tissue, favouring bacterial invasion and chronic infection.

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The expression of gingival epithelial junctions in response to subgingival biofilms

G.N. Belibasakis [1*], Jeannette I. Kast [2], Thomas Thurnheer [1], Cezmi A. Akdis [2], Nagihan Bostanci [3]

[1] Oral Microbiology and Immunology, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Switzerland

[2] Swiss Institute of Allergy and Asthma Research (SIAF), University of Zürich, Davos, Switzerland

[3] Oral Translational Research, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Switzerland

* Corresponding Author:

Prof. Georgios N. Belibasakis

Institute of Oral Biology, Center of Dental Medicine

University of Zürich,

Plattenstrasse 11, 8032 Zürich, Switzerland

e-mail: george.belibasakis@zzm.uzh.ch

Tel: +41-44-6343306

Fax: +41-44-6343090

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Running title: Effect of oral biofilm on gingival epithelial junction expression

Abstract

Periodontitis is an infectious inflammatory disease that destroys the tooth-supporting tissues. It is caused by the formation of subgingival biofilms on the surface of the tooth. Characteristic bacteria associated with subgingival biofilms are the Gram-negative anaerobes *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, collectively known as the "red complex" species. Inter-epithelial junctions ensure the barrier integrity of the gingival epithelium. This may however be disrupted by the biofilm challenge. The aim of this *in vitro* study was to investigate the effect of subgingival biofilms on the expression of inter-epithelial junctions by gingival epithelia, and evaluate the relative role of the red complex. Multi-layered human gingival epithelial cultures were challenged with a 10-species *in vitro* subgingival biofilm model, or its variant without the red complex, for 3 h and 24 h. A low-density array microfluidic card platform was then used for analysing the expression of 62 genes encoding for tight junctions, gap junctions, adherens junctions, and desmosomes. Although there was a limited effect of the biofilms on the expression of tight, adherens and gap junctions, the expression of a number of desmosomal components was affected. In particular, Desmoglein-1 displayed a limited and transient up-regulation in response to the biofilm. In contrast, Desmocollin-2, Desmoplakin and Plakoglobin were down-regulated equally by both biofilm variants, after 24 h. In conclusion, this subgingival biofilm model may down-regulate selected desmosomal junctions in the gingival epithelium, irrespective of the presence of the "red complex". In turn, this could compromise the structural integrity of the gingival tissue, favouring bacterial invasion and chronic infection.

Introduction

Periodontal diseases are caused by microbial biofilms that colonize the tooth surfaces and instigate an inflammatory response by the juxtaposed gingival tissue. The microbial species constituting these biofilms are part of the endogenous oral microbiota. Shifts in the tissue micro-environmental conditions may favor the uncontrolled growth of certain species, which now act as pathobionts by establishing a dysbiotic interaction with the host [1-4]. The initial host response is a biological mechanism aimed at preventing bacterial colonization and establishment [5]. Yet, in the case of dysbiosis [1, 6], an excessive inflammatory response may cause tissue destruction, which manifests as periodontitis [7]. The development of a "subgingival" biofilm is a primary etiological agent of a dysbiotic host response. In classical studies, the increase in numbers and proportions of the tree "red complex" species (*Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*) in subgingival biofilms has been highly associated with the presence of periodontitis [8].

The epithelium of the gingival sulcus or periodontal pocket is a first line of defence against the developing biofilm, by constituting a physical barrier, secreting chemo-attractants for neutrophils and permitting their trafficking to the site where the biofilm is established [9-11]. The integrity of all epithelial tissues is ensured by several cell-to-cell molecular adhesion and sealing complexes, including tight junction, adherens junctions, gap junctions and desmosomes [12-14]. The expression of gap and tight junctions has been well documented in the gingiva [15-17]. Therefore, unimpaired expression of these molecular complexes in gingival epithelial tissues is crucial for maintaining their integrity. Once tissue integrity is disturbed by biofilm-derived noxious stimuli, the associated bacteria may be permitted to invade into the deeper

periodontal tissue, triggering an inflammatory response and establishing chronic infection.

Therefore, this study aimed to investigate the effects of an *in vitro* 10-species subgingival biofilm model (designated as "BF") on the gene expression of all known tight junctions, desmosomes, gap junctions and adherens junctions, in a multi-layered gingival epithelial cell culture [18-20], by using a low-density array microfluidic card platform [14, 21]. A further aim was to evaluate the involvement of the three "red-complex" species in the observed effects, by excluding them from the composition of the biofilm (designated as "BF-RC"). This *in vitro* experimental system resembles rather closely the *in vivo* interface between the gingival epithelium and the microbial biofilm.

Results

The effect of BF or BF-RC on the gene expression of tight-, gap- and adherens-junctions by multi-layered gingival epithelial cultures was investigated. Prior to that, it was confirmed that there was no significant quantitative difference in the individual bacterial composition between the two biofilm variants, with the obvious exception of the three "red complex" species, which had been omitted from the inoculum of BF-RC [19]. In addition, it was confirmed that neither BF, nor BF-RC elicited any strong cytotoxic effects on these gingival epithelial cultures [19].

Among the 30 tight junction genes studied, the most highly expressed by the gingival epithelial cells were, sequentially, Claudin-4, Claudin-1, JAM-1, Claudin-25, Claudin-17, Occludin and Claudin-12 (Figures 1A and 2A). On the contrary, Claudin-8, Claudin-18, Claudin-19, Claudin-20 and JAM-2 were not expressed in this epithelial culture under any of the experimental conditions. The effect of the biofilm

challenge was further considered on the regulation of the expressed genes, after 3h and 24 h. It was found that BF did not affect the expression of any of the studied tight junction genes. Absence of the "red complex" from the biofilm (BF-RC) resulted in significantly higher Claudin-4 expression compared to the control or the BF at 3 h (Figure 1A), whereas at 24 h its expression was significantly higher only compared to the control group (Figure 2A). Although these up-regulations proved to be significant, they were rather low numerically, ranging at increases of 14%-36% over the control.

The gene expression of desmosomes, adherens junctions, and gap junction proteins was further determined (Figures 1B and 2B). Only Connexin 32 (GJB1) was not expressed, whereas Desmoglein-2, Desmoglein-4, and Nectin-3 were expressed at low levels. The most highly expressed ones were Desmocollin-2, Desmoglein-1, Desmoglein-3, Connexin 26 and Connexin 43. After 3 h of challenge, Desmocollin-2 expression was significantly up-regulated in response to BF-RC, compared to BF or to the control, by approximately 20% (Figure 1B). However, after 24 h, this was significantly down-regulated by both biofilm variants, by approximately 40%, compared to the unchallenged control (Figure 2B). Desmoglein-1 was significantly up-regulated at 3 h by approximately 44% only in response to BF, but its expression resumed control levels after 24 h (Figure 2B).

All studied junctional adaptor proteins were expressed by the gingival epithelial cultures. Most highly expressed were Desmoplakin, Plakoglobin, and Plakophilin-1. After 3 h of biofilm challenge, the gene expression of none of these proteins was regulated (Figure 1C). However, after 24 h, the expression of Desmoplakin and Plakoglobin were significantly down-regulated in response to both biofilms by approximately 40% and 34%, respectively, whereas there were no significant differences between the two biofilm groups (Figure 2C).

Discussion

The present study investigated the effect of *in vitro* multi-species subgingival biofilms on intra-epithelial junctions expression in multi-layered human gingival epithelial cultures, and evaluated the relative effects of the "red complex" species. While the development of this experimental model is highly relevant for studying the initial tissue responses associated with the pathogenesis of periodontal diseases [22], its potential limitation is that the biofilm comprises of relatively few cultivable species. This may under-represent the full diversity of the cultivable and uncultivable *in vivo* oral microbiome, given that a single periodontal pocket may foster more than a hundred different species [23], and that a dysbiotic environment induces multiple changes in the behaviour of the constituent species [1, 3, 24].

The rationale for this study is that intra-epithelial junctions are crucial for the integrity of the gingival tissue and consequently for the homeostasis and healthy status of the periodontium. Therefore, disruption of their expression may be detrimental for tissue integrity and bacterial invasion. In support of this, recent observations in the present experimental model showed that increased colonization (and potential invasion) of the superficial multi-layered gingival epithelium is associated with disruption signs of the epithelial cell borders, and nuclear degradation [18]. Moreover, a recent proteomic analysis of the secreted proteins in this experimental model showed that several of the down-regulated biological processes and networks are associated with disruption of epithelial tissue integrity and impaired tissue turnover [20].

Among the 30 tight junction genes studied here, only Claudin-4 was affected by the biofilm lacking the three "red complex" species (BF-RC). Yet, the magnitude

of this regulation was rather limited, and may thus not confer any biological relevance. Although Claudin-4 is expressed in healthy and diseased gingival epithelial tissue [25-27], there is as yet no evidence of its regulation by periodontal pathogens. In another experimental model using the same low-density microfluidic card assay, Claudin-4 expression was lower in air-liquid interface nasal epithelial cell cultures from chronic rhinosinusitis patients, than healthy individuals [21].

None of the adherens or gap junction proteins' gene expression was regulated by the subgingival biofilm challenge in the present experimental system. However, the gene expression of two demosomal proteins, namely Desmocollin-2 and Desmoglein-1 were affected. In particular, Desmocollin-2 expression displayed a short-lived and weak up-regulation in response to BF-RC only, but after 24 h this was down-regulated by both biofilm variants, irrespective of the presence of the "red complex". This reduced expression may denote compromised gingival tissue coherence and integrity. To our knowledge, there is at present no further information on the expression of Desmocollin-2 in the healthy or diseased periodontal tissues. Desmoglein-1 expression also displayed a short-lived but significant induction in response to BF at 3 h, which resumed control levels after 24 h. Desmoglein-1 is expressed by the healthy gingival epithelium [28], whereas its expression is down-regulated in the periodontitis affected gingival tissue [29].

Among the junctional adaptor proteins studied, the gene expression of only Desmoplakin and Plakoglobin, two desmosomal-associated proteins, were regulated. Desmoplakin expression has been demonstrated in the gingival epithelium [30-32], whereas Plakoglobin is known to structurally associate with Desmoglein-1 [33]. To date, there has been no study on the effects of the biofilm on the expression of these two proteins in gingival epithelium. The present study demonstrated that after 24 h of

challenge with either biofilm, the expressions of both Desmoplakin and Plakoglobin were significantly reduced. Once again, this down-regulatory trend may denote an active loss of tissue integrity. Since, the regulatory effect of the two biofilms variants was of similar magnitude, the "red complex" may not hold a crucial role in this event.

At this stage, a comparison with *in vivo* studies is worth considering. For instance, an immunohistochemical study using biopsies from clinically healthy gingiva and advanced periodontitis lesions demonstrated reduced E-cadherin, involucrin, Connexin 26 and Connexin 43 staining in the epithelial lining of the periodontal pocket, associated with alterations of filamentous actin expression [34]. Hence, that study concluded that the profound perturbation of the lining epithelium in periodontitis compromises its ability to function as an effective barrier against microbial invasion. Although a different set of junctions was affected in the present *in vitro* epithelial tissue-biofilm interaction model, the findings point to a similar direction, namely the down-regulation of junctions necessary for tissue integrity. While *in vivo* studies provide direct insights into changes within the periodontitis-affected tissues, *in vitro* models such as the one employed here, can give answers to mechanistic questions, due to their highly controlled and reproducible nature. As such, we were able to show that the "red complex" species had minimal interference in junctions gene expression.

Finally, it should be acknowledged that this study screened for broad transcriptional changes in epithelial junctions expression, rather than their regulation on the protein level. The findings may allude to proteins that could be investigated in more detail. Collectively it is shown that the present subgingival biofilm model used as a polymicrobial challenge did not cause major alterations in the gene expression of tight, gap or adherens junctions over an experimental period of 24 h. Nevertheless, it

down-regulated the expression of three desmosome-associated proteins, and this was not commensurate with the presence of the three "red complex" species. Hence, subgingival biofilms may down-regulate the transcription of selected desmosomal junctions in the gingival epithelium, an effect that may compromise structural tissue integrity and enable bacterial invasion, should this also prove to translate on the protein level *in vivo*.

Materials and methods

In vitro biofilm model

The 10-species *in vitro* "subgingival" biofilm model used in this study was grown as previously described [19, 35, 36]. It consisted of the individual species *Campylobacter rectus* (OMZ 697), *Fusobacterium nucleatum* (OMZ 598), *P. gingivalis* ATCC 33277^T (OMZ 925), *Prevotella intermedia* ATCC 25611^T (OMZ 278), *T. forsythia* OMZ1047, *T. denticola* ATCC 35405^T (OMZ 661), *Veillonella dispar* ATCC 17748^T (OMZ 493), *Actinomyces oris* (OMZ 745), *Streptococcus anginosus* (OMZ 871), and *Streptococcus oralis* SK 248 (OMZ 607). This biofilm variant is referred to as "BF", while its 7-species variant lacking *P. gingivalis*, *T. forsythia* and *T. denticola* (i.e. the "red complex") is referred to as "BF-RC" in the manuscript text. These biofilms were grown in 24-well cell culture plates on sintered hydroxyapatite discs, in order to mimic the natural tooth-biofilm interface. The hydroxyapatite discs were pre-conditioned for 4 h with 800 µl of pasteurized human saliva diluted 1:1 in sterile saline, in order to establish a pellicle on their surface. Biofilm formation was initiated by inoculating on the pellicle-covered hydroxyapatite 1.6 ml of growth medium consisting of 60% saliva, 10 % heat-inactivated human serum, 30 % modified fluid universal medium (mFUM) [36, 37] with 0.3 % glucose, and 200 µl of a bacterial cell

suspension containing equal volumes and densities from each strain. The volumes were not adjusted according to the size of each strain in the suspension. After 16.5 h of anaerobic incubation at 37 °C, the medium was replenished, and 50 µl of *T. denticola* liquid culture were also added (OD_{550} = 1.0). Biofilms were grown anaerobically for further 48 h and during this period, the discs were "dip-washed" in saline three times daily for 1 min, and the medium was replenished once daily. After a total 64.5 h of incubation, the biofilm-grown hydroxyapatite discs were carefully placed onto the multi-layered gingival epithelial cell cultures (described below), mediated by a plastic ring to ensure a distance of 1 mm, and co-cultured for 3 h or 24 h. These time-points represent an earlier and a later host response to the biofilm. At each one of these two time-points, the discs were removed from the cultures and subsequently processed for analysis of bacterial composition by quantitative real-time Polymerase Chain Reaction (qPCR), as previously described [19, 36]. Three independent biofilms were performed per each experimental group. Pellicle pre-coated hydroxyapatite discs were used as controls. This pellicle derived from the same saliva batch and was processed according to the same protocol as the biofilm grown-discs, but omitting the bacterial suspensions. Three independent cell cultures were performed in each experimental group.

Cell cultures

Stratified multi-layered gingival epithelial cell cultures in 24-well plate format (0.5 cm^2 surface) were used (EpiGing, MatTek, Ashland, MA, USA) and maintained in culture in defined keratinocyte serum-free medium, supplemented with 0.05 mM calcium chloride and 200 mM L-glutamine (Gibco/Invitrogen, Lucerne, Switzerland). These cultures resemble morphologically the gingival epithelium, as they comprise of

normal human gingival epithelial cells forming a highly differentiated multi-layered tissue with keratinized layers.

RNA extraction and cDNA synthesis

After completion of the experiments, the culture supernatants were removed and the multi-layered gingival epithelia were washed twice in phosphate buffer saline. Thereafter, they were lysed and total RNA was extracted by using the RNeasy Mini Kit (Qiagen). The concentration of the RNA was measured by a NanoDrop 1000 spectrophotometer (Thermoscientific). One µg of total RNA was then reverse transcribed into single-stranded cDNA by M-MLV Reverse Transcriptase, $Oligo(dT)_{15}$ Primers, and PCR Nucleotide Mix (Promega), at 40°C for 60 min, and 70°C for 15 min. The resulting cDNA was stored at -20°C.

Gene expression analysis by TaqMan low-density array microfluidic cards

A total of 62 predesigned gene expression assays (Applied Biosystems) representing the junctional apparatus of epithelial cells were selected for the analyses performed in this study (Table 1) [14, 21]. The probes were spanning over an exon-exon junction and amplified an amplicon length of maximal 200 nt. As housekeeping gene, GAPDH was used (Applied Biosystems assay ID: Hs99999905-m1). From the extracted total RNA, 400 ng were used per microfluidic card, and the reactions were run in a 7900HT Fast Real-Time PCR System (Applied Biosystems), using a TaqMan Universal PCR MasterMix (Applied Biosystems, 4304437). Arbitrary units representing gene expression were calculated with the following formula: arbitrary units = $2^{(-\Delta ct)}$ x 1000. Genes whose transcription was undetectable beyond 40 cycles under any of the experimental conditions were considered as non-expressed.

Statistical analysis

A two-way analysis of variance (ANOVA) was used to analyze the statistical significance of differences, using Tukey's test for multiple comparisons between groups. Differences were considered statistically significant at $P<0.05$.

Disclosure

The authors declare no conflicts of interest with respect to authorship and/or publication of this article.

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Tables

Table 1

The 62 genes included in the microfluidic card mRNA expression array.

Gene symbol	***Gene name***	***Assay ID***	***Expressed***
OCLN	Occludin	Hs00170162_m1	Yes
F11R	JAM-1	Hs00170991_m1	Yes
JAM2	JAM-2	Hs00221894_m1	No
JAM3	JAM-3	Hs00230289_m1	Yes
MARVELD2	Tricellulin	Hs00376394_m1	Yes
CLDN1	Claudin-1	Hs01076359_m1	Yes
CLDN2	Claudin-2	Hs00252666_s1	Yes
CLDN3	Claudin-3	Hs00265816_s1	Yes
CLDN4	Claudin-4	Hs00976831_s1	Yes
CLDN5	Claudin-5	Hs00533949_s1	Yes
CLDN6	Claudin-6	Hs00607528_s1	Yes
CLDN7	Claudin-7	Hs00600772_m1	Yes
CLDN8	Claudin-8	Hs00273282_s1	No
CLDN9	Claudin-9	Hs00253134_s1	Yes
CLDN10	Claudin-10	Hs00199599_m1	Yes
CLDN11	Claudin-11	Hs00194440_m1	Yes
CLDN12	Claudin-12	Hs01082669_m1	Yes
CLDN14	Claudin-14	Hs00273267_s1	Yes
CLDN15	Claudin-15	Hs00204982_m1	Yes
CLDN16	Claudin-16	Hs00198134_m1	Yes
CLDN17	Claudin-17	Hs01043467_s1	Yes

CLDN18	Claudin-18	Hs00212584_m1	No
CLDN19	Claudin-19	Hs00381204_m1	No
CLDN20	Claudin-20	Hs00378662_m1	No
CLDN23	Claudin-23	Hs01013638_s1	Yes
CLDND1	Claudin-25	Hs00219886_m1	Yes
TMEM114	Claudin-26	Hs00418203_m1	Yes
C1orf91	Claudin-27	Hs00963921_m1	Yes
ASAM	CMLP	Hs00293345_m1	Yes
GJA1	Connexin-43	Hs00748445_s1	Yes
GJB1	Connexin-32	Hs00939759_s1	No
GJB2	Connexin-26	Hs00955889_m1	Yes
GJB6	Connexin-30	Hs00917676_m1	Yes
GJC3	Connexin-29	Hs01384570_m1	Yes
CDH1	E-cadherin	Hs01023895_m1	Yes
PVRL1	Nectin-1	Hs01591978_m1	Yes
PVRL2	Nectin-2	Hs01071562_m1	Yes
PVRL3	Nectin-3	Hs00210045_m1	Yes
DSG1	Desmoglein-1	Hs00355084_m1	Yes
DSG2	Desmoglein-2	Hs00170071_m1	Yes
DSG3	Desmoglein-3	Hs00170075_m1	Yes
DSG4	Desmoglein-4	Hs00698286_m1	Yes
DSC1	Desmocolin-1	Hs00245189_m1	Yes
DSC2	Desmocolin-2	Hs00951428_m1	Yes
DSC3	Desmocolin-3	Hs00170032_m1	Yes
MPDZ	MUPP1	Hs00187106_m1	Yes

TJP1	ZO-1	Hs01551876_m1	Yes
TJP2	ZO-2	Hs00910541_m1	Yes
TJP3	ZO-3	Hs00274276_m1	Yes
CGN	Cingulin	Hs00430426_m1	Yes
CGNL1	Paracingulin	Hs00262671_m1	Yes
MAGI1	MAGI-1	Hs00191026_m1	Yes
MAGI3	MAGI-2	Hs00326365_m1	Yes
INADL	PATJ	Hs00195106_m1	Yes
MARVELD3	MARVELD3	Hs00369354_m1	Yes
JUP	Plakoglobin	Hs00158408_m1	Yes
DSP	Desmoplakin	Hs00189422_m1	Yes
PKP1	Plakophilin-1	Hs00240873_m1	Yes
PKP2	Plakophilin-2	Hs00428040_m1	Yes
PKP3	Plakophilin-3	Hs00170887_m1	Yes
PKP4	Plakophilin-4	Hs00269305_m1	Yes
CTNNB1	B-catenin	Hs00355049_m1	Yes

The gene symbols, gene names and gene expression assay IDs are provided, as well as the information whether they were expressed (Yes/No) in the present experimental model by the multi-layered gingival epithelial culture.

JAM, Junctional adhesion molecule; MAGI, membrane-associated guanylate kinase inverted; MUPP1, multi-PDZ domain containing protein 1; ZO, zonula occludens.

Figure legends

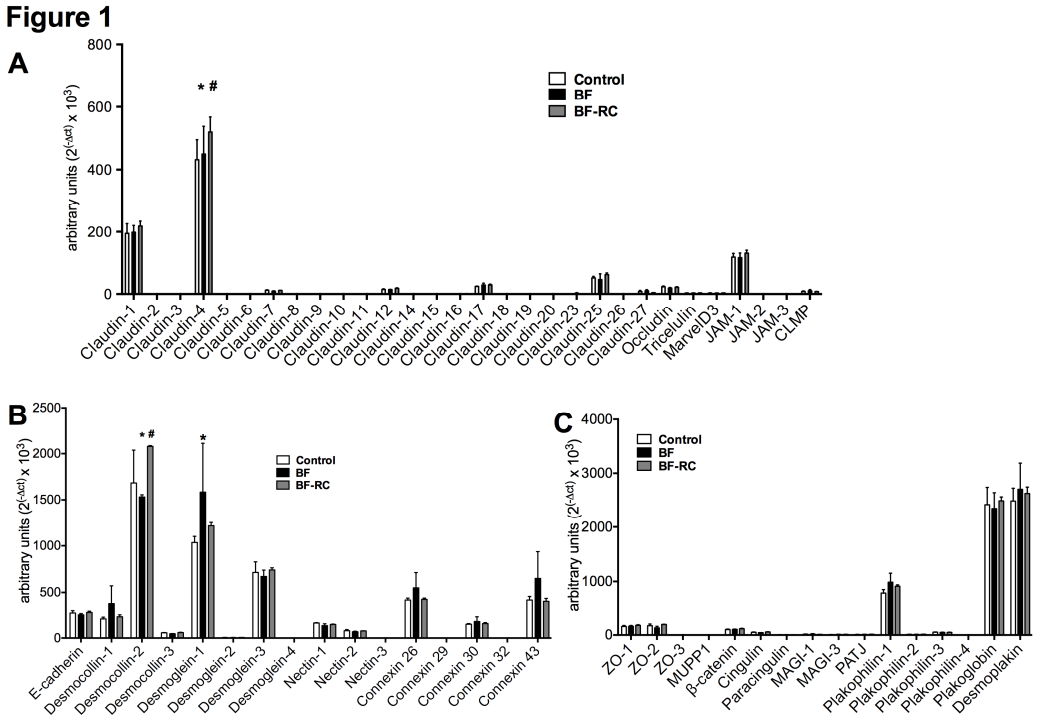

Figure 1. Junctions gene expression profile in multi-layered gingival epithelial cells cultures, assayed by Taqman low-density array microfluidic card. The cell cultures were challenged for 3 h with BF or BF-RC, and thereafter the gene expression of transmembrane tight junction proteins (A), desmosomes, adherens junctions, and gap junction proteins (B), as well as junctional adaptor proteins (C) were assayed. Bars represent mean values ± SEM from three independent cell cultures in each group. Two-way ANOVA was used to calculate the differences between groups. Asterisks (*) represent statistically significant difference compared to the control group, whereas hash tags (#) represent statistically significant difference compared BF (P<0.05).

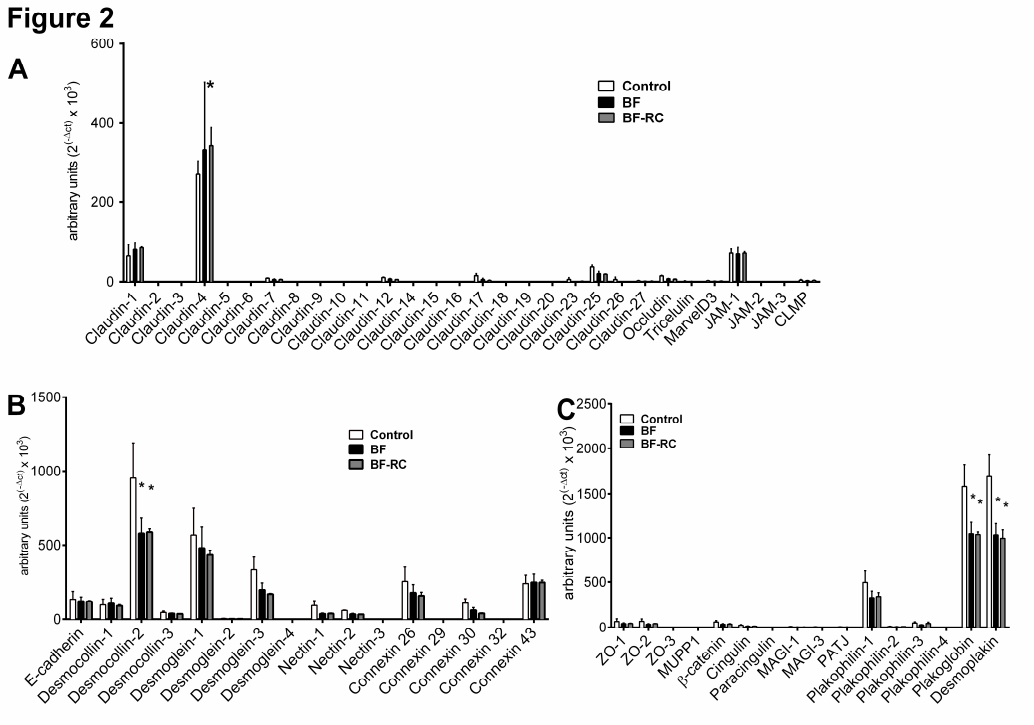

Figure 2. Junctions gene expression profile in multi-layered gingival epithelial cells cultures, assayed by Taqman low-density array microfluidic card. The cell cultures were challenged for 24 h with BF or BF-RC, and thereafter the gene expression of transmembrane tight junction proteins (A), desmosomes, adherens junctions, and gap junction proteins (B), as well as junctional adaptor proteins (C) were assayed. Bars represent mean values ± SEM from three independent cell cultures in each group. Two-way ANOVA was used to calculate the differences between groups. Asterisks (*) represent statistically significant difference compared to the control group, whereas hash tags (#) represent statistically significant difference compared BF (P<0.05).